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Investigation of belinostat-induced genomic instability by molecular cytogenetic analysis and pathway-focused gene expression profiling

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ABSTRACT

Histone deacetylases (HDACs), which regulate transcription and specific functions such as tumor suppression by p53, are frequently altered in tumors and have a contentious role in carcinogenesis. HDAC inhibitors, which have a long history of use in psychiatry and neurology, have recently been tested as possible treatments for tumors. Belinostat received regulatory approval in the USA on July 3, 2014, for use against peripheral T-cell lymphoma. However, the unavailability of information on belinostat genotoxicity in normal cells and the molecular mechanisms involved in the genetic instability after exposure to belinostat encouraged us to conduct this study. Our data showed that the exposure of mice to belinostat at the recommended human doses induced chromosome breakage, whole-chromosome lagging, and oxidative DNA damage in bone marrow cells in a dose-dependent manner. The expression levels of 84 genes involved in the DNA damage signaling pathway were evaluated by using an RT^2 Profiler PCR array. Belinostat exposure altered the expression of 25 genes, with statistically significant changes observed in 17 genes. The array results were supported by RT-PCR and western blotting experiments. Collectively, our results showed that belinostat exposure caused oxidative DNA damage and downregulated the expression of genes involved in DNA damage repair, which may be responsible for belinostat-induced genomic instability. Thus, the clinical usage of this drug should be weighed against the hazards of carcinogenesis, and the observed genotoxicity profile of belinostat may support further development of efficient HDAC inhibitors with weaker genotoxicity.

1. Introduction

Histone deacetylases (HDACs) belong to a class of enzymes implicated in the remodeling of chromatin. They play an important role in the epigenetic regulation of gene expression (Choudhary et al., 2009). HDACs, which are overexpressed in many cancer types, play significant roles in mediating aberrant epigenetic reprogramming, silencing of tumor suppressor genes such as p53, and favoring transformation (Bolden et al., 2006; Pan et al., 2007). As an additional mode of action through which HDACs contribute to the cancerous phenotype, they eliminate acetyl residues from non-histone proteins and alter their function. The alteration of histones through epigenetic dysregulation is an important oncogenic mechanism that has been exploited in tumor drug development. Recently, the inhibition of HDACs has been developed as a potential strategy to invert aberrant epigenetic alterations that accompany tumors. Numerous classes of HDAC inhibitors (HDACi) were observed to have powerful and specific antitumor properties in preclinical experiments. The anticancer property of HDACi has been associated with their ability to induce gene expression through the acetylation of histone and non-histone proteins.

The targeting of HDAC activity by therapeutic small-molecule HDACi has emerged as an exciting therapeutic approach (Marks et al., 2004). There is a long history of the use of HDACi as psychiatric pharmaceutical drugs for mood disorders. Currently, they are being studied as potential anti-tumor compounds. Moreover, they have demonstrated potential as immunosuppressant, anti-inflammatory, anti-neurodegenerative, and antiparasitic compounds (Mwakwari et al., 2010). HDACi are classified into numerous groups based on their chemical structure, such as those with hydroxamic acids (*e.g.*, panobinostat, vorinostat, and belinostat), short-chain fatty acids (*e.g.*, valproate and sodium butyrate), aminobenzamides (*e.g.*, entinostat and mocetinostat), cyclic peptides (*e.g.*, apicidin and romidepsin), epoxyketones

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(*e.g.*, trapoxins), and hybrid molecules. To date, the approved HDACi for the treatment of peripheral T-cell lymphoma are vorinostat, romidepsin, and, recently, belinostat (Lee et al., 2015; Rashidi and Cashen, 2015).

In addition to the three FDA-approved agents, newer second-generation HDACi, for example panobinostat, pracinostat, and entinostat, have been extensively tested in clinical settings, with varying therapeutic results. However, none of them have yet been registered for clinical use (Nebbioso et al., 2012; Qiu et al., 2013). Although the associated toxicities are largely controllable, the cardiotoxicity and hematological and gastrointestinal effects can be dose limiting (Gryder et al., 2012). HDACi induce tumor cell cycle arrest, differentiation, and cell death, reduce angiogenesis, and alter immune response. The mechanisms through which HDACi exhibit antitumor properties are not consistent; they may depend on the type of tumor, the individual HDACi, and the applied dose (Eckschlager et al., 2017).

Genomic instability refers to various DNA changes, from single nucleotides to whole-chromosome alterations. Therefore, it is a driving force of carcinogenesis, as continuous change in the cellular genome encourages the acquisition of further DNA changes, clonal evolution, and cancer heterogeneity (Sieber et al., 2003). In several cases, it is difficult to locate information on the genotoxic potentials of HDAC drug candidates with a hydroxamate group that have been advanced to clinical trials. Nevertheless, the genotoxic evaluation of the three approved HDACi (belinostat, vorinostat, and panobinostat) has been presented in the pharmacological review(s) submitted to the USFDA (Shen and Kozikowski, 2016). Vorinostat was mutagenic in the Ames test and clastogenic in CHO cells, but not in normal human lymphocytes; it showed positive effects in the mouse micronucleus test (Kerr et al., 2010). Both belinostat and panobinostat were found to be mutagenic in the Ames test. Furthermore, in genotoxicity experiments with the L5178YTK$^{+/-}$ mouse lymphoma cell mutagenesis system, belinostat and panobinostat induced clastogenicity in mammalian cells in the presence or absence of metabolic activation. Belinostat was shown to be genotoxic in the *in vivo* clastogenicity assay and in the rat bone marrow micronucleus assay.

On July 3, 2014, the FDA granted accelerated approval for belinostat (Beleodaq; Spectrum Pharmaceuticals, Inc.) for the management of patients with relapsed or refractory peripheral T-cell lymphoma (Lee et al., 2015). Belinostat has shown potent anticancer activity; compared with other HDACi, it appears to have tolerable side effects, although hematological toxicity is rare (Eckschlager et al., 2017). Despite the mixed results of these clinical evaluations, the well-tolerated side effects of belinostat have encouraged the study of combination regimens. At present, most of the data on the genotoxic potential of belinostat have been obtained from unpublished experiments conducted by Spectrum Pharmaceuticals Company and have only been used for registration or marketing purposes (Center for Drug Evaluation and Research, 2014). In addition, these unpublished experiments provided results based on conventional tests and did not present a quantitative evaluation of damage and repair to genetic materials or other molecular data on the mechanism(s) of genotoxicity. Thus, a detailed evaluation of the genomic instability of belinostat in normal cells and the molecular mechanisms underlying its genotoxicity is urgently warranted. Such evaluations are important as genomic damage, particularly in the population of undifferentiated cells, is perilous as it can increase the spontaneous mutation rate and lead to gross alterations in genetic material. The risks of secondary malignancy and other drug-related tumors could be increased by belinostat treatment if such cells survive and proliferate.

2. Materials and methods

2.1. Animals

The investigations were conducted using adult male Swiss albino mice, aged 12–15 weeks and weighing 22–28 g. The mice were acquired from the Experimental Animal Care Center at King Saud University and housed under a 12 h light–dark cycle. The mice diet consisted of rodent standard pellet food and drinking water *ad libitum*. The studies were conducted in accordance with the Guidelines of the Animal Care and Use Committee of King Saud University, Kingdom of Saudi Arabia. All animals were allowed to acclimatize in plastic cages (six mice/cage) inside a well-ventilated room for 7 days prior to the study. Each treatment and control group consisted of a minimum of six mice. After drug treatment, the mice were provided access to food and drinking water *ad libitum* until they were killed by cervical dislocation.

2.2. Micronucleus test and FISH analysis of micronuclei

Belinostat (Selleck Chemicals Company, USA) was dissolved in 10% dimethylsulfoxide (DMSO) in normal saline and administered *via* a single intraperitoneal injection of 50, 100, 200, or 300 mg/kg/day for 2 days consecutively. Mouse bone marrow cells were collected 24 h after the final injection. The control animals received equal amounts of 10% DMSO in saline. The administered volume was 0.01 mL/g body weight. The administered doses of belinostat were chosen on the basis of their efficiency in inducing clastogenesis *in vivo* (Center for Drug Evaluation and Research, 2014). The standard dosage of belinostat in humans is 1000 mg/m^2, administered a as a single agent, once daily, on days 1–5 of a 21-day cycle. Mice have a body weight-to-surface area ratio of approximately 3 kg/m^2. Thus, the dose of 200 mg/kg was equivalent to approximately 600 mg/m^2. In the current experiment, one of the six mice (16.6%) treated with 300 mg/kg belinostat died within 3 h of exposure. Two additional groups of animals were intraperitoneally injected with 2 mg/kg of the positive control clastogen mitomycin C (Sigma-Aldrich, St. Louis, MO) or 2 mg/kg of the positive control aneugen colchicine (Sigma-Aldrich, St. Louis, MO) and killed after 24 h (Attia, 2011). Bone marrow cells were flushed from both femurs into glass tubes containing fetal bovine serum. Subsequently, the smears of bone marrow cells were prepared on microscopic slides (Attia et al., 2005). A minimum of four microscopic slides were prepared for each mouse and allowed to dry at 37 °C overnight. For each mouse, one slide was stained with May-Grunwald and Giemsa solutions for the conventional assessment of micronucleated polychromatic erythrocytes (MNPCEs) in addition to bone marrow suppression, as previously described (Attia, 2007; Attia, 2009). From each slide, 1000 PCEs were examined for MNPCEs by using a light microscope. In addition, the number of PCEs among 1000 NCE/slide was counted to assess bone marrow suppression. The other three slides were maintained at −20 °C to differentiate between the clastogenic and aneugenic properties of belinostat through the recognition of the origin of micronuclei (MN) by a fluorescence *in situ* hybridization (FISH) technique using a labeled centromeric DNA probe, as previously described (Attia, 2009). The presence of centromeric-signals in the MNPCEs was analyzed by using a fluorescence microscope, and approximately 100 MN/group of coded microscopic slides were scored for the absence or presence of the centromeric signals.

2.3. Assessment of DNA strand breakage and oxidative DNA damage by standard and modified comet assay

The kinetics of DNA damage repair were detected in the bone marrow cells by using a standard comet assay based on the OECD guidelines. Belinostat was administered *via* a single intraperitoneal injection of 50, 100, or 200 mg/kg/day for 2 days consecutively, and mice were killed at 3 and 24 h after the final injection of belinostat. The positive control animals were intraperitoneally injected with 50 mg/kg *N*-Ethyl-*N*-nitrosourea (ENU; Sigma-Aldrich, St. Louis, MO) (Attia et al., 2014), and mouse bone marrow cells were collected 24 h after ENU injection. The cells were flushed from one femura into glass tubes with fetal bovine serum. Subsequently, 10 μL (approximately 10,000 cells) of